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VITALITY OF THE GAMETES OF *CUMINGIA TELLINOIDES.*

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Cumingia tellinoides is a small lamellibranch mollusk found abundantly in the Woods Hole region. Its breeding season extends over a period of three or three and a half months, beginning early in June. The production of eggs is continuous and spawning by each mature individual occurs two or three times during the course of the breeding season. Eggs may be had in great abundance during this time. One female may shed 300,000 eggs, although the average number extruded is estimated to be between 100,000 and 200,000. They are expelled into the sea water where they are fertilized.

The present paper is a report on experiments to determine the vitality of the gametes, and more especially the longevity of the unfertilized eggs. It may be assumed that when eggs are released from the ovary, they are normal. From the time of liberation, deterioration begins and if not fertilized they ultimately go to pieces by fragmenting in a characteristic manner. The extensive work of Goldforb indicates that some deterioration takes place in sea urchin eggs while stored in the ducts of the gonad before extrusion. This may also be true of *Cumingia*, since two clear cases of physiologically poor lots of eggs have been found among hundreds of normal ones. Deterioration o both gametes of *Cumingia* and of *Arbacia* is rapid after extrusion into sea water.

Vitality of the Eggs.

The eggs used in these experiments were obtained by isolating sexually mature individuals in small stender dishes filled with sea

water into which the eggs were shed. The eggs, so collected in one dish, may therefore be conveniently referred to in the description of the experiments as a "batch" or a "lot" of eggs. The reference in all cases is to the eggs of a single female.

During the summer of 1926 many batches of eggs were kept without fertilization to test their longevity. During the progress of the experiments a few eggs were taken from the dishes from hour to hour and fertilized as a test of their physiological condition and of the rate of deterioration as indicated by decreasing percentage of fertilization. The experiments reported here deal principally with the longevity of the unfertilized eggs as indicated by the time of disintegration.

Approximately two per cent. of the eggs of most females are defective at spawning and fragment within four or five hours. The other ninety-eight per cent. remain intact and capable of development for longer periods. The poorest lot of eggs when tested at a temperature of 20° to 22° centigrade went to pieces completely in six hours, and it was not uncommon for 98 per cent of the eggs of some females to fragment in from eight to nine hours. The great majority of the lots of eggs fragmented in from nine to fifteen hours, the average being between ten and twelve hours. When tested at 18.5° to 20° C., on the other hand, the most vigorous batches of eggs, remained intact from fifteen to twenty hours, that is, the eggs retained their normal appearance for that length of time. It was shown that a majority of the eggs either refuse to develop or develop abnormally, when fertilized shortly before fragmentation. However, a few normal embryos come from the oldest eggs, even when the percentage of fertilization is greatly reduced. When a lot of eggs is fertilized after fifty per cent. of them have fragmented, it not infrequently happens that most of those which remain intact fertilize and cleave. However, most of them die in cleavage or gastrula stages and few develop into normal embryos. The oldest lots of eggs to develop normally were ten and twelve hours old and rarely fifteen hours old. These all showed 95 to 98 per cent. fertilization and normal development.

The extreme limits of longevity of the unfertilized eggs are therefore found to be six to twenty-six hours, and the limits of

fertilization and normal development in the best lots of eggs are ten to fifteen hours. These figures are for temperatures normally experienced by the eggs of the species, or 18.5° to 22° C. They therefore represent the normal longevity of the eggs.

The statement by Morgan that the eggs of *Cumingia* will not stand rough handling is misleading. They are injured by centrifuging but there is abundant evidence that they develop normally under ordinary laboratory manipulation.

Tables I., II. and III. deal with various phases of the longevity and vitality of the eggs and include representative experimental data.

Individual Variation.

After any particular lot of eggs begins to fragment, three or four hours elapse before all or 98 per cent. have fragmented. The variation in longevity within a single group is, therefore, approximately four hours. For example, if 10 per cent. of the eggs have fragmented at twelve hours, the average expectancy of fragmentation for the remainder would be 30 per cent. in thirteen hours, 50 per cent. in fourteen, 70 per cent. in fifteen, and 95 to 98 per cent. in sixteen hours. Two per cent. may remain intact indefinitely. The rate of fragmentation is shown in Table I., as is also an actual comparison of the longevity of five lots of eggs from five females studied under identical conditions. Tables II. and III. show the same thing in slightly different form.

When fresh eggs are fertilized by fresh sperm the percentage of cleavage is usually between 97 and 100 per cent. However, a few lots of eggs gave from 89 to 95 per cent. cleavage. One lot of physiologically poor eggs was observed which when fertilized with physiologically good sperm gave only 20 to 30 per cent. cleavage. This sperm when used to fertilize other lots of eggs gave from 97 to 100 per cent. cleavage showing that failure to cleave on the part of these eggs was not due to defective sperm. This lot of physiologically poor eggs was slimy from the first and showed a tendency to cling to the containing dish. This together with one other lot of defective eggs may be regarded as confirmation of Goldforb's contention that eggs at the time of spawning may show all the symptoms characteristic of aging

Table I.

Vitality of *Cumingia* Eggs.

Record of five lots of eggs spawned by five females on August 16 and kept unfertilized at 20° C. The vertical columns, when read from top to bottom, give the result of eleven examinations that were made with the microscope at approximately hourly intervals in order to ascertain the time that elapsed before fragmentation began in each lot of eggs as well as the rate of fragmentation. The percentages refer to the number of eggs that remain normal in appearance. From three to five hundred eggs were counted in each examination.

	♀ No. 1.	♀ No. 2.	♀ No. 3.	♀ No. 4.	♀ No. 5.
After 5½ hrs.	100% normal	97% normal	100% normal	100% normal	100% normal
After 6½ hrs.	70% normal	100% normal	100% normal	100% normal	100% normal
After 8 hrs.	20% normal	5% normal	100% normal	100% normal	100% normal
After 9 hrs.	0% normal	0% normal	100% normal	100% normal	98% normal
After 10 hrs.			100% normal	100% normal	98% normal
After 11½ hrs.			100% normal	98% normal	90% normal
After 12 hrs.			100% normal	98% normal	80% normal
After 13 hrs.			98% normal	95% normal	50% normal
After 14 hrs.			95% normal	85% normal	30% normal
After 14½ hrs.			90% normal	70% normal	2% normal
After 15 hrs.			80% normal	50% normal	0% normal

Comment.—Eggs from lots 3, 4 and 5 were inseminated with freshly shed sperm after 11½ hours. The percentage fertilization which resulted was estimated to be 90 to 95 per cent. and abundant normal larvæ developed. Many eggs are apparently normal for 12 hours after extrusion. Eggs from lot 3 were fertilized 13 hours after extrusion which gave 75 per cent. cleavage and normal development. It appears that there is great variation in the vitality of the eggs of different females. These five lots spawned by five different females during the same hour were treated alike. One fragmented in seven hours, one in eight hours, one in fourteen hours, one in sixteen hours and it may be predicted in the light of former experience that the other would have fragmented completely in seventeen or eighteen hours if the experiment had been continued. No. 1 began to fragment in six hours. This gives the range of variation in longevity among the eggs from the five females as six to eighteen hours and normal fertilization for twelve hours in the best of the five. These five lots of eggs are substantially typical and may be considered as representative, although some are considerably longer lived than those used in this experiment. At least twenty such experiments were carried out during the season. The extreme limits of longevity were found to be from five to twenty-six hours. Only one or two per cent. of the best lots of eggs remained intact after the expiration of that time. It is interesting to compare this with the egg of *Arbacia* which, according to Goldforb and others, has a longevity of 40 to 48 hours.

TABLE II.

NORMAL VARIATION IN PERCENTAGE CLEAVAGE IN THE EGGS OF *Cumingia tellinoides*.
Temperature 22° C.

August 4. Freshly spawned eggs from ten females were fertilized by the sperm of one male to show the initial variation in the percentage of cleavage of eggs of this species. This table also shows the effect of aging. It is interesting to note that after 90 to 95 per cent. of the eggs have fragmented those remaining intact may fertilize and cleave. (Compare Tables II. and III.) All eggs normal and fragmented, are included in the calculation of the percentages.

	After 2 Hrs.	After 8 Hrs.	After 11 Hrs.	After 14 Hrs.	After 16 Hrs.
Female No. 1.....	98% cleavage	98% cleavage	93% cleavage	10% cleavage	0% cleavage
Female No. 2.....	98% cleavage	98% cleavage	40% cleavage	0% cleavage	0% cleavage
Female No. 3.....	92% cleavage	10% cleavage	0% cleavage	0% cleavage	0% cleavage
Female No. 4.....	97% cleavage	70% cleavage	71% cleavage	50% cleavage	2% cleavage
Female No. 5.....	98% cleavage	7% cleavage	0% cleavage	0% cleavage	0% cleavage
Female No. 6.....	96% cleavage	97% cleavage	92% cleavage	60% cleavage	20% cleavage
Female No. 7.....	99% cleavage	98% cleavage	96% cleavage	2% cleavage	0% cleavage
Female No. 8.....	95% cleavage	95% cleavage	95% cleavage	40% cleavage	10% cleavage
Female No. 9.....	88 to 90% cleavage	30% cleavage	20% cleavage	1% cleavage	0% cleavage
Female No. 10.....	30% cleavage	0% cleavage	0% cleavage	0% cleavage	0% cleavage

eggs. The first vertical column of Table II. shows the usual range of variation in freshly spawned eggs in respect to percentage fertilization and cleavage.

Table III.

Longevity of the Eggs of *Cumingia tellinoides*, Temperature 22° C.

The eggs of the ten females compared in Table II. in respect to percentage fertilization are here compared as to longevity and time of fragmentation or disintegration. It will be noted that the time of reduction in cleavage is almost synchronous with the time of fragmentation. This relationship does not always hold because in numerous experiments the percentage of cleavage fell off before fragmentation. In other words, if the reduction in cleavage due to aging and the rate of fragmentation were plotted as curves they would not as a rule be superimposed or parallel.

	Percentage of Fragmentation with Aging.			
	After 7 Hrs.	After 11 Hrs.	After 15 Hrs.	After 16 Hrs.
Female No. 1.....	0%	0%	50%	100%
Female No. 2.....	0%	45%	100%	100%
Female No. 3.....	75%	100%	100%	100%
Female No. 4.....	0%	2%	72%	90%
Female No. 5.....	73%	100%	100%	100%
Female No. 6.....	0%	0%	40%	50%
Female No. 7.....	0%	0%	99%	100%
Female No. 8.....	0%	0%	12%	60%
Female No. 9.....	0%	7%	100%	100%
Female No. 10.....	100%	100%	100%	100%

Note.—Many of the eggs of lots 4, 6 and 8 lived beyond sixteen hours. The other lots of eggs had fragmented completely after sixteen hours.

Vitality of the Spermatozoa.

The work of Gemmill ('00) and F. R. Lillie ('15) has shown that variations in the concentration of sperm suspensions of *Arbacia* make a great difference in the duration of their fertilizing ability. The writer accordingly used several sperm dilutions in order to learn whether the same phenomena are exhibited by the sperm of *Cumingia*.

Spermatozoa shed by a mature male *Cumingia* of average size in 30 cc. of sea water was estimated by actual measurement to be a 2 to 3 per cent. suspension.[1]

[1] The method of measuring the percentage was to kill the spermatozoa by the addition of formalin and after settling they were measured en masse. From this the calculation of the concentration of the original suspension was a simple matter.

This concentrated suspension, though at best somewhat variable, was used as a standard suspension from which various dilutions were made. It was found possible to select suspensions of approximately the same strength and this is a matter of more importance than that the exact percentage be known.

Two drops[2] of standard sperm suspension when added to 25 cc. of sea water was estimated to be a 1/500 to 1/750 per cent. suspension; two drops in 50 cc. of sea water was considered to be a 1/1000 to 1/1500 per cent. suspension; one drop in 50 cc. of sea water makes a 1/2000 to 1/3000 per cent. suspension, etc. The last named suspension when fresh is adequate to fertilize one hundred per cent. of the eggs whereas greater dilutions sometimes gave only partial fertilization. What was estimated to be a 1/4000 to 1/6000 per cent. suspension gave from eighty to one hundred per cent. fertilization and usually one hundred per cent. All of these suspensions were used and also the same percentage suspensions in larger quantities of sea water.

The method of studying the relative longevity of these various dilute sperm suspensions was to make up, by the proper dilutions, several dishes of each from freshly shed sperm. To these fresh eggs were added in turn at hourly intervals until the suspensions no longer gave fertilizations. It was shown in general that the weakest suspensions die first. Tables 4 and 5 show that the longevity of the spermatozoa depends largely upon the degree of concentration. Even a suspension of 1/500 per cent. shows some preserving effect in that spermatozoa live for a longer time in this concentration than in greater dilutions. There is little difference between a 1/2000 and a 1/6000 per cent. suspension, and these no doubt represent natural conditions so far as longevity is concerned.

In work reported at this time the writer had in mind to study the longevity of gametes under natural conditions. In general the longevity of the sperm in the most dilute suspensions is somewhat less than that of the eggs. In a few cases using suspensions of 1/500 per cent. approximately ninety per cent. normal embryos developed from sperm and eggs that were

[2] The same pipette was used in making all dilutions of an experiment and one giving approximately one cc. per 20 drops

twelve hours old and in numerous other cases at nine and ten hours. As a rule when sperm suspensions of 1/2000 to 1/3000 per cent. are used very few fertilizations occur after nine or ten hours. Numerous experiments show that spermatozoa begin to die after three and one half or four hours. The indication therefore is that a majority of the spermatozoa under natural conditions live from four to nine hours.

Table IV.

Longevity of the Sperm of *Cumingia* in Various Dilutions as Shown by the Percentage of Fertilizations that they Give with Fresh Eggs (July 25).

Age of the Suspension Tested.	1 Drop in 100 Cc. Sea Water 1/4000 to 1/6000%.	1 Drop in 50 Cc. Sea Water 1/2000 to 1/3000%.	4 Drops in 50 Cc. Sea Water 1/500 to 1/750%.	8 Drops in 50 Cc. Sea Water 1/250 to 1/500%.
2 hours	99%+	99%+	100%	100%
3½ hours	96%	95%	97%	96%
7 hours	17%	24%	99%	97%
10 hours	2%	8%	63%	72%

For this experiment the spermatozoa used were all from the same male. The eggs were from three females and were not over three hours old when used. Controls showed them to be practically 100 per cent. normal eggs.

Table V.

Longevity of the Sperm of *Cumingia* in Various Dilutions Tested on July 23.

Sperm all from one male.

Age of Sperm Suspension Used.	1 Drop in 50 Cc. Sea Water 1/2000 to 1/3000%.	2 Drops in 50 Cc. Sea Water 1/1000 to 1/1500%.	4 Drops in 50 Cc. Sea Water 1/500 to 1/750%.	6 Drops in 50 Cc. Sea Water 1/300 to 1/500%.
2¼ hours	84% cleaved	100% cleaved	100% cleaved	99%+ cleaved
4⅚ hours	35% cleaved	65% cleaved	75% cleaved	80% cleaved
7½ hours	15–20% cleaved	30% cleaved	50–60% cleaved	85–87% cleaved
9 hours	0% cleaved	0% cleaved	2% cleaved	35–40% cleaved

This table shows that sperm in 1/2000 to 1/3000 per cent. suspension died in nine hours. Other experiments have shown that from ten to forty per cent. of the spermatozoa in these most dilute suspensions often survive for 10 to 12 hours. Table IV. is more typical in this respect, but even in this case the percentage of fertilization is below the average. The point that is shown clearly is that spermatozoa die first in the most dilute suspensions and that a suspension of 1/500 to 1/750 per cent. suspension preserves the life of the sperm beyond their normal life in the open sea.

When the work of Gemmill and Lillie is taken into consideration it is apparent that Goldforb's experiments on the aging of spermatozoa do not represent normal conditions. He apparently relied upon concentrated dry sperm which are known to age much more slowly than sperm kept under natural conditions. The aging of sperm in concentrated suspensions has no particular significance. His work on the eggs is certainly not open to the same criticism but I should question his interpretation and results on the aging of spermatozoa. The work of the other two authors and this present work show that the life of spermatozoa in dilute suspensions is brief.

The statement by Lillie that the spermatozoa of *Arbacia* lose ability to fertilize eggs in a few minutes in dilute suspensions is not verified for *Cumingia* sperm, although the theory that they gradually lose something to the water which is essential to fertilization and which ultimately renders them incapable of bringing about the fertilization reaction may very well be true.

It has been my observation that spermatozoa that are able to swim actively are capable of fertilizing eggs; and that they do not lose the ability to initiate the fertilization reaction after a few minutes as reported by Lillie for *Arbacia*. Up to the present time no attempt has been made to learn whether or not the spermatozoa of *Cumingia* contain an activating substance, essential to fertilization. In many ways the spermatozoa of *Cumingia* behave as those of *Nereis* and *Arbacia* do, but if they contain a sperm receptor (Lillie, 15) preliminary experiments indicate that it is dissipated in the sea water more slowly than in the cases investigated by Lillie. Experiments on this question are in progress, as well as investigations of the cause of excessive polyspermy. These experiments are designed for comparison of the gametes of *Cumingia*, *Chætopleura* and *Hydroides* with the work of Goldforb and Gemmill on sea urchin eggs and with Lillie's work on the spermatozoa of *Nereis* and *Arbacia*.

Activation of Spermatozoa.

Spermatozoa, after having apparently lost their vitality, may be revived. When placed in a dish with eggs they become activated and swim vigorously. The activation of sperm in the

presence of eggs was observed repeatedly. The stimulus from the eggs or egg water is evident almost instantly, but not all of the spermatozoa are so activated. I interpret this to mean that most of the quiescent spermatozoa are already dead or weakened beyond the possibility of functioning.

The phenomenon of activation of apparently spent sperm by the exudations from eggs is interesting and not uncommon. The physiological value of this activation is evident although the real cause is obscure. Lillie (13) shows that the spermatozoa of *Nereis* and *Arbacia* are positively chemotropic to weak acids and to egg secretions and are apparently stimulated by them.

Concentrated Sperm Suspensions.

It was learned that spermatozoa in concentrated suspensions retain their vitality for very long periods. Under such conditions they may swim from twenty-four to thirty-six hours and give 90 to 100 per cent. fertilization and normal development. They retain sufficient life for four days to show occasional contractions visible under a compound microscope. It is evident therefore that there is some protective element in this unnatural concentration. Cohn claims that it is carbon dioxide or hydrogen ion concentration. Dry sperm of fishes has the same extended longevity although possibly from different causes, not having had the initial stimulus to swim and use up its limited store of energy.

Discussion.

Goldforb expresses the belief that deterioration of eggs begins at the time of maturation while they are still stored in the gonads. He attributes the great variability of sea urchin eggs to the differences in time that they remain in storage before spawning takes place. I find a similar variation in the vitality of eggs of *Cumingia*, but it is noteworthy that maturation does not take place in the eggs of this species until after the entrance of the spermatozoön which occurs after extrusion into the sea water. Deterioration in the gonad in this case, therefore, could not be due to maturation. The fact that eggs vary so much in their longevity leads to the belief that some deterioration takes place before extrusion and before maturation. It is hardly likely that

the great difference in vitality that experiment reveals is due entirely to natural variability. The eggs are stored in the gonads and their ducts for some time before extrusion and those that are stored longest may very well show physiological deterioration. Two cases of eggs which showed all the symptoms of aging at spawning verify Goldforb's contention on this point. At the present time I am unable to state whether the considerable variability in the longevity of *Cumingia* eggs is due principally to normal variability or is in part due to physiological deterioration while in storage. Cases of low percentage fertilization in the eggs of *Cumingia* are rare. As a rule they give 97 to 100 per cent. cleavage, although the longevity may be more variable.

Summary.

I. The average longevity of *Cumingia* eggs when kept at a temperature of 20° to 22° C. is 10 to 12 hours as judged by time of fragmentation and ability to give a high percentage of normal embryos. The average longevity at 18.5° to 20° C. is 12 to 15 hours.

II. Approximately two per cent. of any lot of eggs may be defective as shown by their early fragmentation which occurs long before the rest.

III. The outstanding fact is the wide range of variation in the vitality and longevity of *Cumingia* eggs. The eggs of a single individual vary by four hours or approximately 25 per cent., while the eggs of different females vary in their longevity from six to twenty-six hours or over 400 per cent.

IV. Eggs in rare instances show deterioration at spawning, apparently due to long-time storage in the ducts of the gonads. Most lots of eggs when freshly spawned give from 97 to 100 per cent. cleavage and normal development.

V. The longevity of the best sperm in suspensions of 1/400 to 1/500 per cent. is 10 to 12 hours as judged by functional activity and ability to give 90 to 100 per cent. fertilization and normal development. In sperm suspensions of 1/2000 to 1/3000 per cent. it is 4 to 7 hours, but from 30 to 50 per cent. fertilization may frequently be expected from suspensions 7 to 12 hours old and 1 to 5 per cent. fertilization from suspensions 12 to 20 hours old.

VI. Spermatozoa in concentrated suspensions of 1 to 3 per cent. retain their vigor for many hours. They frequently give 90 to 100 per cent. fertilization after twenty-four to thirty-six hours, and some individual spermatozoa live for four days, still showing faint contractions of the tail at intervals. This preservation of the life of the spermatozoa is attributed to the presence in the water of CO_2 produced by the activity of the spermatozoa, or to hydrogen ion concentration.

VII. Spermatozoa after becoming quiescent show activation in the presence of eggs or egg water.

This paper was read by title before the American Society of Zoölogists at the meeting in Philadelphia, December, 1926. An abstract was printed in the *Anatomical Record.*

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